Blood Coagulation Values in Normal Sheep and in Two Mutant Strains with Hyperbilirubinemia

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SUMMARY

Blood coagulation values for normal sheep and lambs, using commercially available reagents for human coagulation, are reported. Of the domestic animal species for which coagulation values have been reported, the sheep most closely resembles those of humans. Factor VII deficiency has been found in some patients with Dubin Johnson syndrome. Sheep with the same defect were found to have no coagulation abnormality.

RÉSUMÉ

Les auteurs rapportent les valeurs de coagulation sanguine de moutons et d'agneaux sains; ils ont déterminé ces valeurs à l'aide de réactifs commerciaux utilisés pour la détermination des mêmes valeurs chez les humains. Parmi les espèces d'animaux domestiques dont on a rapporté les valeurs de coagulation, le mouton est celui dont ces valeurs ressemblent le plus à celles de l'homme. On a noté un déficience du Facteur VII chez des patients atteints du syndrome de Dubin Johnson. Les moutons souffrant de ce syndrome ne manifestaient pas d'anormalité de coagulation.

INTRODUCTION

Acquired Factor VII deficiency which is commonly associated with liver injury in humans (1,8,15,18,21), has recently been observed in patients with Dubin Johnson syndrome (17). One mutant strain of Corriedale sheep is the only domestic animal known to have this disease (1,4). Hyperbilirubinemia due to a defect in organic anion uptake, similar to that in Crigler-Najjar syndrome in man, has been observed in Southdown sheep (5,6,12). Both strains of sheep affected with hyperbilirubinemia were examined for coagulation defects because of the association of liver disorders and Factor VII deficiency in man.

This paper presents detailed blood coagulation values (not found in the literature) for normal mature sheep and normal lambs, as well as for the two strains of sheep having hyperbilirubinemia.

MATERIALS AND METHODS

Blood coagulation studies were made on 55 sheep: (a) 21 normal mature Corriedale adults (two males, 19 ewes); (b) 18 normal mature Southdown adults (two males, 16 ewes); (c) four normal lambs (two males, two females); (d) four normal Southdown lambs (two males, two females); (e) six mature Corriedale sheep (all ewes) with Dubin Johnson syndrome; and (f) two mature Southdown sheep (males) with congenital bilirubin-uptake defect.

Sheep with hepatic transport defects were kept in shaded barns on straw bedding because they suffer from photosensitive dermatoses. The normal sheep and lambs were intensively husbanded. All sheep were fed a free choice pelleted ration, good quality hay and water *ad libitum*.

Blood was withdrawn from the jugular vein by venipuncture into a plastic sterile syringe and either mixed with 0.1 M sodium oxalate to give a final dilution of 10:1 or allowed to clot. Plasma was separated from the cells as quickly as possible. Serum was harvested after incubation of each clotted blood sample for two hours at 37°C. Plasma and serum were stored at

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TABLE I. Fibrinogen Concentration, Prothrombin Time (PT), Prothrombin and Proconvertin Time (PP), Partial Thromboplastin Time (PTT), and Thromboplastin Generation Clotting Times (TGT) (at Two, Four, Six and Eight Minutes after Activation of Mixture) for Blood from Normal Adult Sheep and Lambs, Adult Sheep with Dubin Johnson Syndrome and Southdown Adult Sheep with Congenital Hyperbilirubinemia.

	Normal Adult Sheep	Normal Lambs	Sheep with Dubin Johnson Syndrome	Southdown Sheep with Hyperbilirubinemia
Number of Animals	**39**	**8**	**6**	**2**
Fibrinogen (mg/100)	440 ± 58	375 ± 62	379 ± 15	429
Plasma PT (secs)	14.7 ± 1.0	15.1 ± 1.5	15.2 ± 1.2	13.2
Serum PT (secs)	40.9 ± 15.2	53.1 ± 14.8	55.2 ± 7.2	—
PP (secs)	43.8 ± 4.2	55.0 ± 7.3	51.1 ± 4.9	39.6
PTT (secs)	41.1 ± 8.7	55.7 ± 12.7	52.3 ± 5.8	31.1
TGT (secs)				
2 minutes[a]	19.7 ± 6.4	34.0 ± 9.1	23.5 ± 5.2	8.1
4 minutes	17.2 ± 4.7	30.4 ± 4.5	16.0 ± 4.8	15.2
6 minutes	17.8 ± 4.9	30.6 ± 6.2	14.2 ± 3.1	16.0
8 minutes	19.6 ± 6.6	30.7 ± 6.7	15.4 ± 4.5	17.8

[a]Time after thromboplastin generation

0–4°C until tested. All coagulation tests were performed within four hours after obtaining blood specimen. No samples having a serum or plasma hemoglobin concentration greater than 50 mg/100 ml were studied. Sheep absorbed plasma was prepared by adding 100 mg barium sulfate, U.S.P., to 1 ml of fresh plasma and then centrifuging the mixture. The prothrombin time of the absorbed plasma exceeded 180 seconds in all cases.

Fibrinogen (10), plasma prothrombin time (plasma PT) (16), partial thromboplastin time (PTT) (19), serum prothrombin time (serum PT) (14), prothrombin and proconvertin time (PP) (23), and thromboplastin generation time (TGT) (3) were measured by standard procedures using commercial reagents[1] and a coagulation timer[2]. Hemoglobin concentration of blood (11), serum or plasma (7) were measured. Hematocrit was determined using microhematocrit method.

Reconstituted, lyophilized human plasma was included with each batch of determinations to serve as a control[3]. Strict attention was given to standardizing incubation at 37°C because it was found that overheating (42°C) sheep serum during incubation markedly influenced the TGT results.

TGT is an index of the sufficiency of Factors V, VIII, IX, X, XI and XII required in the first phase of hemostasis. To generate thromboplastin activity, prothrombin-free absorbed plasma, serum, platelets and calcium were combined, and to initiate clotting, prothrombin and fibrinogen were added to the mixture. Clotting occurs quickly in the presence of adequate thromboplastin but is delayed if any one of or a combination of the above-mentioned coagulation factors is present in limited quantity. TGT clotting time of normal human absorbed plasma and serum, is less than 16 seconds at either two, four or six minutes of incubation. Although this clotting time is of similar magnitude to that of plasma PT, the two tests are independent. The choice of 15 seconds to differentiate normal from abnormal TGT values for sheep was therefore, arbitrary.

RESULTS

All sheep were clinically healthy and showed no bleeding, bruising or hypercoagulation tendencies. The two Southdown sheep with hyperbilirubinemia had lower packed cell volume (averaging 27%) and lower hemoglobin concentration (9.2 g/100 ml) than did the other sheep (packed cell volume averaging 38.0 ± 3.0%, and hemoglobin concentration 13.1 ± 1.4 g/100 ml).

Table I presents coagulograms for nor-

[1]FibroPlastin Rabbit Brain Thromboplastin with Calcium, BBL No. 40776, FibroLet Activated Platelet Factor Reagent, Lyophilized, BBL No. 40780, Thromboplastin Generation Kit, BBL No. 40640, Baltimore Biological Laboratories, Division of BioQuest, Baltimore, Maryland.

[2]Fibrometer Precision Coagulation Timer, BBL No. 60115, Baltimore Biological Laboratories, Division of BioQuest, Baltimore, Maryland.

[3]FibroTrol Normal Coagulation Control Plasmas, Lyophilized, BBL No. 40777, Baltimore Biological Laboratories, Division of BioQuest, Baltimore, Maryland.

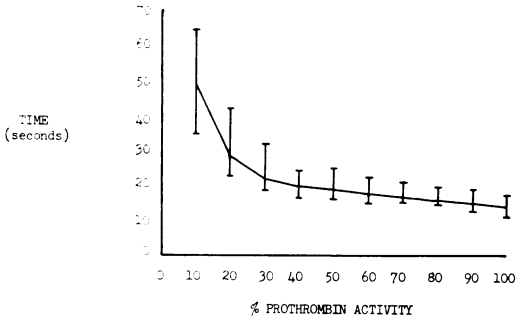

Fig. 1. Normal prothrombin activity curve for mature sheep (mean and range, 23 sheep).

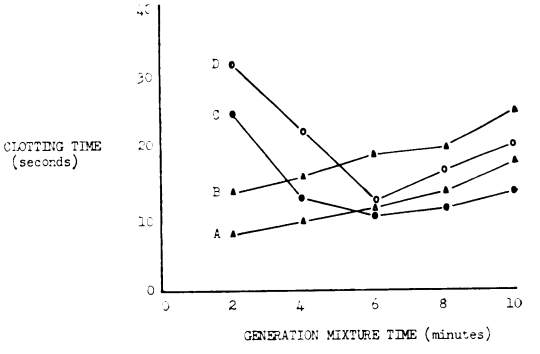

Fig. 2. Normal curves for TGT in sheep. Curves A and B. illustrate the fast clotting occurring after two mins generation; curves C and D illustrate the fast clotting occurring after six mins generation.

mal lambs and mature sheep. Figure 1 presents the normal prothrombin time curve. Normal curves for TGT in sheep are given in Fig. 2. Both strains of sheep with hyperbilirubinemia had blood coagulation values similar to normal sheep.

The coefficient of variation for the TGT values at two, four, six and eight minutes after activation averaged 27.4, 24.0, 23.3 and 38.3% respectively, for all sheep. Because of the wide range, the two-minute TGT data (Table II) were subdivided into four groups to indicate clot-formation time: (a) under ten seconds, (b) 11-20 seconds, (c) 21-30 seconds, and (d) greater than 30 seconds. Fourteen of the 53 sheep (two affected Southdown not included) did not have a TGT clotting time of less than 15 seconds regardless of length of incubation. With some sheep, clotting time was determined after ten minutes incubation; two sheep having slow clotting times after eight minutes of incubation produced faster clotting reactions (12.1 and 12.4 seconds) after ten minutes.

Six lambs and six mature sheep had slow TGT curves; two mature sheep each had a minimum clotting time of 17.8 seconds, and with the other ten sheep the minimum clotting time exceeded 20 seconds (Table III). The six mature sheep with abnormal TGTs had significantly increased plasma and serum PT, PP, and PTT values as compared with the 33 normal mature sheep having normal TGT. The PT increase, however, was not significantly different from the PT of the mature Dubin Johnson sheep, which had normal TGT values. Lambs with abnormal TGT values had prolonged PTT and PP values.

Blood of the lambs with prolonged TGT values had, at two minutes after incubation, clotting times greater than 30 seconds; of the affected mature sheep, four

TABLE II. Analysis of the Thromboplastin Generation Clotting Times (TGT) of 38 Adult Sheep, Six Dubin Johnson Sheep, and Eight Lambs Based on Clotting Time at Two Minutes after Activation of Generation Mixture

		Average Thromboplastin Generation Time (TGT) (secs)			
Grouping Based on Clotting Time Two Minutes after Generation of Thromboplastin Activity	Number of Animals[a]	Two Minutes[b]	Four Minutes	Six Minutes	Eight Minutes
10 secs.....	19	7.3 ± 1.3	9.9 ± 1.8	11.7 ± 1.6	13.9 ± 1.5
11-20 secs.....	10	13.6 ± 1.4	16.2 ± 4.2	19.2 ± 4.7	20.4 ± 4.9
21-30 secs.....	10	27.1 ± 1.7	18.0 ± 3.6	17.1 ± 6.1	17.1 ± 5.4
30 secs.....	14	38.7 ± 3.1	33.6 ± 5.3	31.0 ± 7.1	30.7 ± 6.7

[a]Southdown sheep not included
[b]Time after thromboplastin generation

TABLE III. Average Coagulation Values of Sheep Showing Abnormal Thromboplastin Generation Clotting Times (TGT)

	Adult Sheep	Lambs
Number of Animals	6	6
Plasma PT (secs)	15.6[ab]	14.9
PP (secs)	48.8[b]	61.0[c]
PTT (secs)	59.6[c]	63.9[c]
Serum PT (secs)	101.4[c]	53.7
TGT (secs)		
2 minutes	33.6	39.8
4 minutes	28.5	35.4
6 minutes	26.2	34.6
8 minutes	27.1	34.3

[a]$P < 0.05$
[b]Value significantly different
[c]$P < 0.01$, from comparable values for animals with normal TGT results

had initial TGTs between 21 and 30 seconds and two greater than 30 seconds. Replacement of sheep absorbed plasma with reconstituted, freeze dried, human absorbed plasma provided a more active generation mixture, whereas inclusion of reconstituted, freeze dried, human serum in place of sheep serum had no effect (Table IV). Similarly, replacement of absorbed plasma, and not serum, from sheep with normal TGT values also corrected the defect. TGT curves were probably not the result of an *in vivo* coagulation defect, because blood samples withdrawn from three mature sheep with abnormal TGT curves, 24 hours after the abnormality was recognized, gave normal results. Unfortunately none of the lambs were bled immediately after the abnormal results were found. TGT values were normal in all lambs bled two months later. Thus it would appear that the preparation of the absorbed plasma removed either Factor V or Factor VIII activity, or both.

TABLE V. Average Coagulation Values of 16 Blood Samples Withdrawn from an Adult Sheep at Irregular Intervals over Two and a Half Months

Plasma PT (secs)	14.2 ± 0.27
Serum PT (secs)	34.4 ± 6.8
PP (secs)	52.2 ± 4.2
PTT (secs)	25.4 ± 0.9
TGT (secs)	
2 minutes	6.9 ± 1.6
4 minutes	9.0 ± 2.1
6 minutes	10.5 ± 1.8
8 minutes	12.7 ± 1.6

DISCUSSION

Although some human patients with Dubin Johnson syndrome have specific Factor VII deficiency, acute hepatocellular necrosis is commonly associated with multiple deficiencies of Factors II, VII, IX and X (1,8,18). Because hyperbilirubinemia in these strains of sheep is due to a transport defect, the functional significance is similar to chronic biliary obstruction. The presence of bile in the gastro-intestinal tract is essential for normal absorption of Vitamin K, which in turn is required for the synthesis of Factors II, VII, IX and X. Because of the normal coagulograms in the two strains of sheep with hyperbilirubinemia of different pathogenesis, they apparently have normal Vitamin K absorption.

Newborn animals have a relative deficiency of some blood coagulation factors (23) which normally does not persist. In these experiments, six of the eight lambs, between two and seven weeks old, had prolonged TGT and PTT values suggesting that lambs may not develop the necessary coagulation activity until comparatively

TABLE IV. Sheep with Prolonged Thromboplastin Generation Clotting Time (TGT); Correction of This Abnormality by Addition of Human Absorbed Plasma

Time after Activating Thromboplastin Generation Mixture	Sheep Serum and Plasma	Sheep Serum and Human Absorbed Plasma	Sheep Plasma and Human Serum
2 minutes	48.9 seconds	25.1 seconds	39.2 seconds
4 minutes	50.4 seconds	20.1 seconds	40.4 seconds
6 minutes	46.4 seconds	12.9 seconds	40.9 seconds
8 minutes	44.4 seconds	12.9 seconds	39.9 seconds
10 minutes	41.4 seconds	14.9 seconds	36.9 seconds

late as compared with other mammals; six mature sheep also had prolonged blood coagulation values. The abnormal TGT curves in lambs and mature sheep could be corrected by including human absorbed plasma in the generation mixture. The absorbed plasma supplied Factors V, VIII, XI and XII. As serum also contained Factors XI and XII and did not correct the abnormal TGT, either Factor V or Factor VIII was absent or limited in the absorbed plasma of those sheep with the prolonged TGT curves. All affected animals also had significant increases in PP time; however, when the PP data are expressed as percentage of prothrombin and not in absolute units, the animals have a 70 to 80% normal prothrombin complement. PP clotting time is accepted as being abnormal if it is less than 25% of the normal prothrombin activity. One disadvantage of the standard TGT test is that it does not distinguish between mild and severe Factor VIII deficiency (23). A high level of serum PT activity is found in all deficiencies of phases 1 and 2 of coagulation; if Factor VIII was deficient, then serum PT should be significantly less than plasma PT. In all mature sheep and lambs studied, serum PT was tremendously prolonged as compared with plasma PT. Therefore, the plasma of those animals with an abnormal TGT before the preparation of the barium sulfate absorbed plasma, had adequate Factor V and VIII activity.

It is unlikely that variation in platelet or thromboplastin activity caused abnormal TGT activity, because abnormal and normal results were obtained in the same batch of analyses. Thromboplastin is species specific (9, 22). Because replacement with absorbed plasma from sheep with normal TGT values corrected slow clotting times when the same thromboplastin and platelet factor reagent was used, the coagulation defect was therefore in the absorbed plasma. The lability of Factor V (13) probably accounted for failure to get satisfactory reactions with barium sulfate, U.S.P., as reported previously (20).

Reaction times for blood-coagulation tests in sheep are similar to those in humans. PP values, which are considerably longer in sheep are an exception. The 1:10 plasma dilution used in PP tests increases sensitivity to slight variations in Factor II, V, VII and X activity (23); apparently sheep have less of one of, or perhaps all of these factors than has man.

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